

# **Quantitative Shotgun Proteomics of HD Induced Corneal Injury and Angiogenesis (Briefing Charts)**

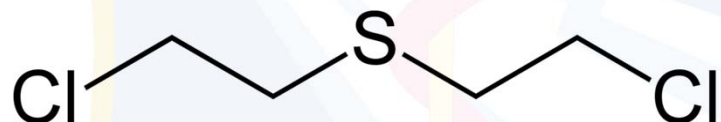


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**Wright Patterson Air Force Base**

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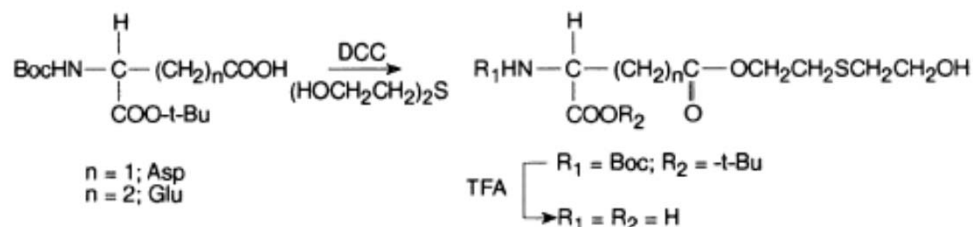


# Sulfur Mustard History and Modification Sites

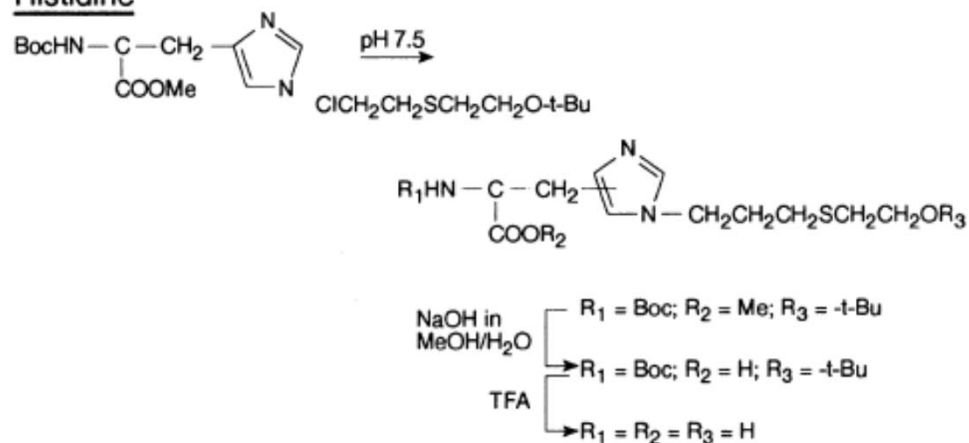


- Used in multiple conflicts in the 20<sup>th</sup> century.
- Causes cornea to become opaque, rendering blindness.
- Attacks DNA and proteins in cells.
- Causes blistering to occur on affected area.
- Non-lethal, but incapacitating to subject.

## Glutamic acid and aspartic acid



## Histidine



Benschop; Arch Toxicol (1997); 71; p171-178

## Mechanism of Sulfur Mustard on Amino Acids

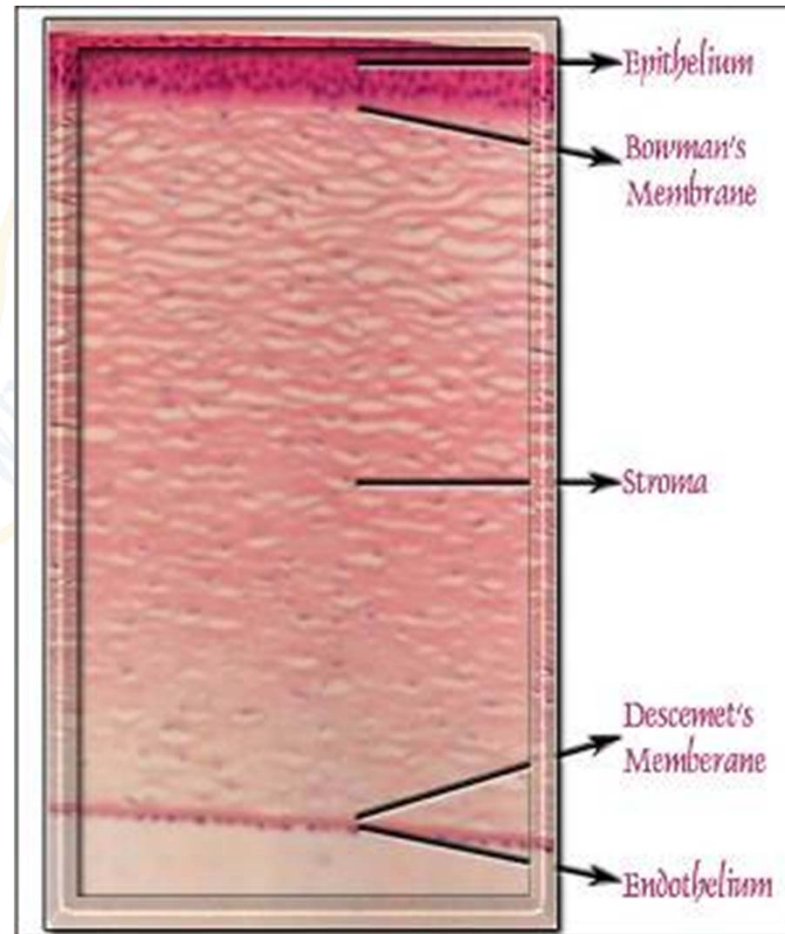




# Layers of the Cornea



- **5 Layers: Epithelium (~50 $\mu$ m), Bowman's Layer (~10 $\mu$ m), Stroma (~450 $\mu$ m), Descemet's membrane (~5-15 $\mu$ m) and Endothelium (~5 $\mu$ m)**
- **Responsible for the majority of refraction of the eye**
- **The stroma is composed mainly of collagen fibrils in differing orientations**
- **Although it accounts for 0.1% of surface area, it is 20 to 50x more susceptible to damage**







# LC-MS/MS vs. 2-D DIGE



## LC-MS/MS

- Requires 1µg of digested mixture
- Analysis time in hours
- Can identify hundreds of proteins in a run
- Up to 8 samples can be analyzed at once

## 2-D DIGE

- Requires 50µg of each sample
- Analysis time in days
- Spots must be excised and analyzed separately by MS/MS





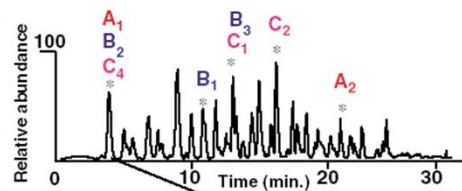
# LC-MS/MS Proteomics

Protein mixture: A, B, C and more

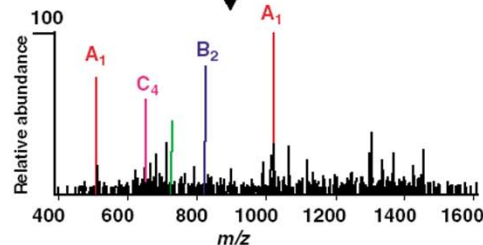
Digest with protease

Peptides: A<sub>1</sub>, A<sub>2</sub>, .....  
B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, .....  
C<sub>1</sub>, C<sub>2</sub>, C<sub>3</sub>, C<sub>4</sub>, ..... and others

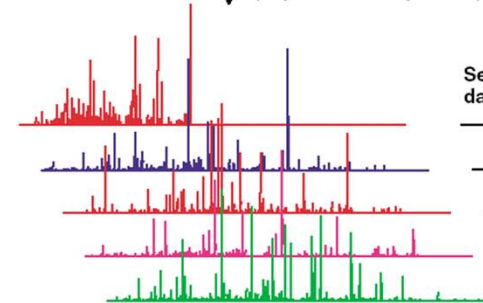
Fractionate by HPLC



Separate by MS



Analyze by MS/MS  
(Top 5 ions, see panel B)



Search  
database

Identification of proteins:  
A, B, C and .....

Sequences of all peptides:

A<sub>1</sub>, A<sub>2</sub>,  
B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>,  
C<sub>1</sub>, C<sub>2</sub>, C<sub>4</sub>, and .....

Output all data

5 peptide sequences:

LLTTIADA  
EFNDPSNAGLQNGFK  
LLTTIADA  
SAGGNYVVFGEAK  
EDDVEEAVQAADR

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Separate Layers / Lyse cells

Digest Proteins

Label with iTRAQ and combine

Separate on UPLC and  
analyze peptides with MS/MS

Identify Proteins and Quantitate



## Instrumentation

Waters Nano-Ultra Performance Liquid Chromatography

- Ultra high pressure LC system capable of separating complex mixtures over long gradient times with no loss in peak width

- Thermo-Finnigan LTQ XL High Performance Linear Ion Trap

- Collision Induced Dissociation (CID) for generating peptide fragmentation

- Pulsed Q Dissociation (PQD) for generating more fragments and extending the low mass range



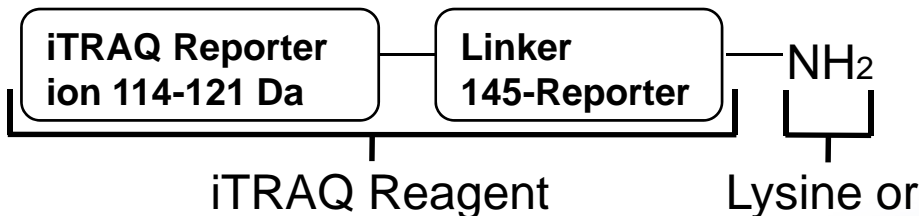
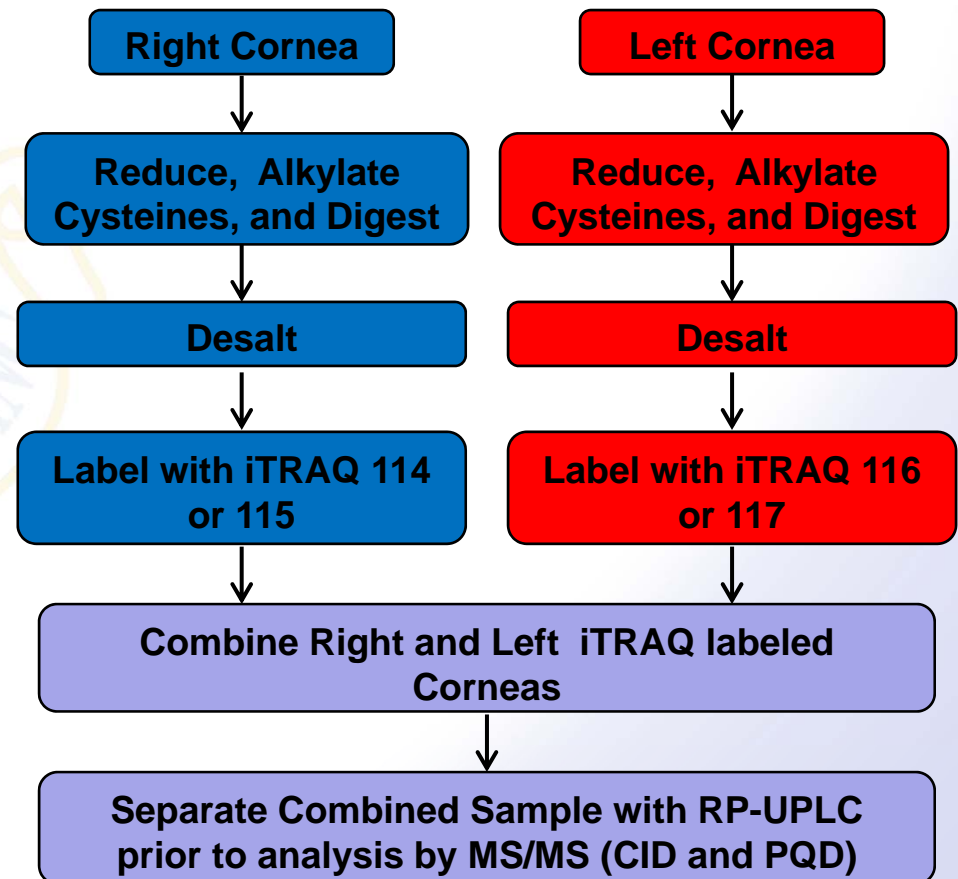


# iTRAQ Labeling for Protein Expression Quantitation



- iTRAQ labels free amine groups (Lysine and N-Terminus), with a mass tag of 144Da
- Peptides elute simultaneously and have the same mass
- During MS/MS fragmentation, the iTraQ is cleaved and reporter ion is recorded as a mass of 114 to 121
- Relative quantitation is achieved by comparing the ratio of the reporter ions

## Workflow of iTRAQ Labeling





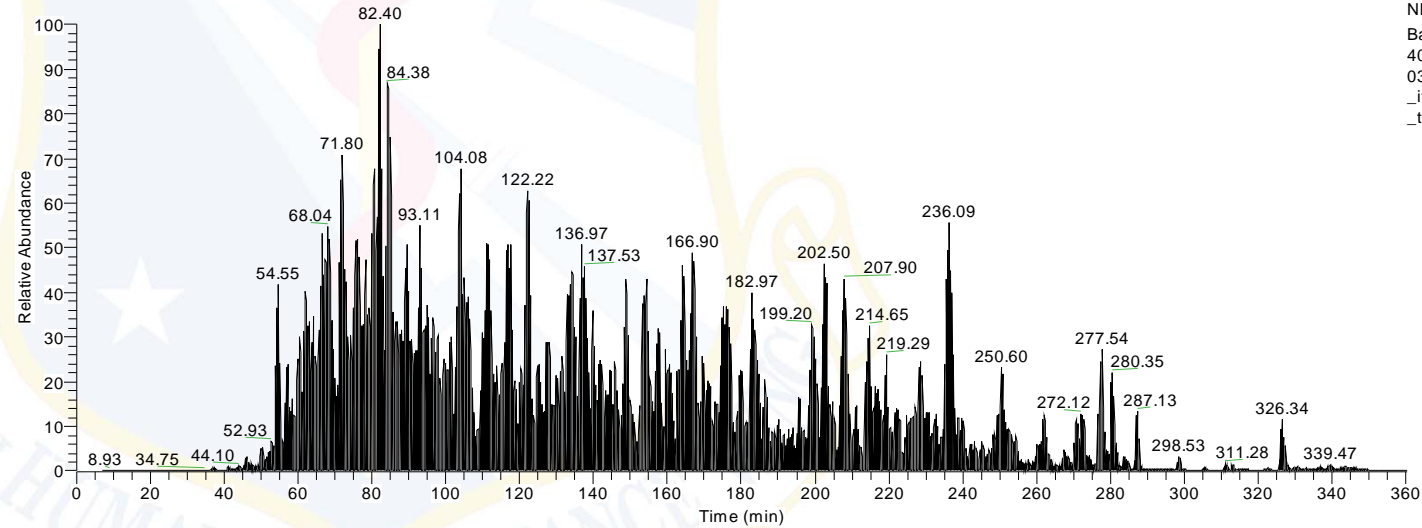
# Quantitative Proteomics with Pulsed Q Dissociation and Collision Induced Dissociation



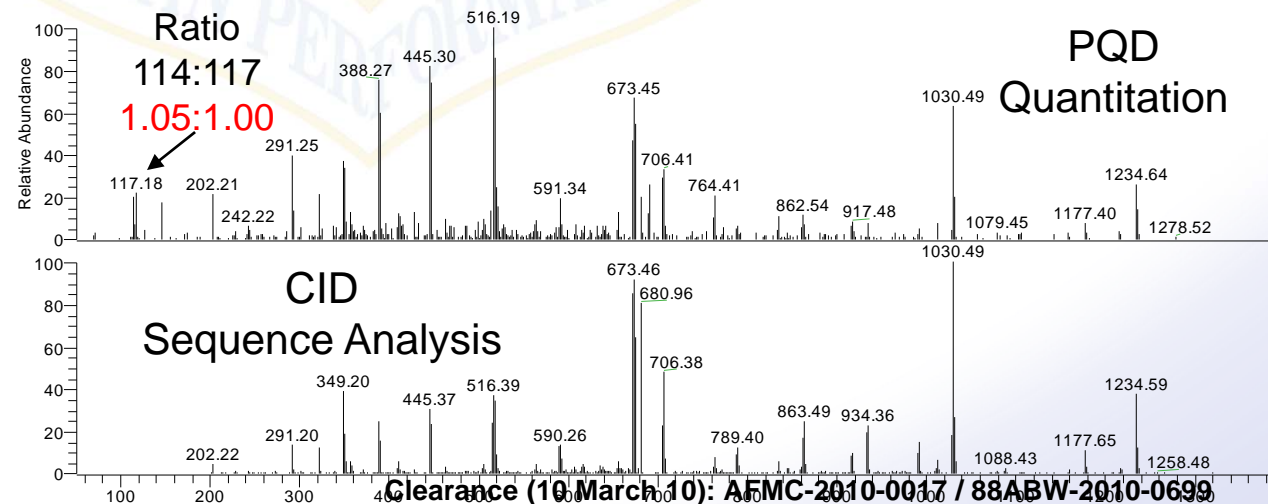
03\_may\_08\_114\_117\_itraq\_Mature\_F2\_2nd...

5/5/2008 10:55:36 AM

RT: 0.00 - 360.05



NL: 2.20E7  
Base Peak m/z=  
400.00-2000.00 MS  
03\_may\_08\_114\_117  
\_itraq\_Mature\_F2\_2nd  
\_trial\_080505105536



NL: 2.60E3  
03\_may\_08\_114\_117\_itraq\_Mature  
\_F2\_2nd\_trial\_080505105536#199  
93 RT: 84.86 AV: 1 T: ITMS + c NSI  
d Full ms2 690.58@ppq35.00  
[50.00-1395.00]

NL: 1.67E4  
03\_may\_08\_114\_117\_itraq\_Mature  
\_F2\_2nd\_trial\_080505105536#199  
94 RT: 84.86 AV: 1 T: ITMS + c NSI  
d Full ms2 690.58@cid35.00  
[180.00-1395.00]



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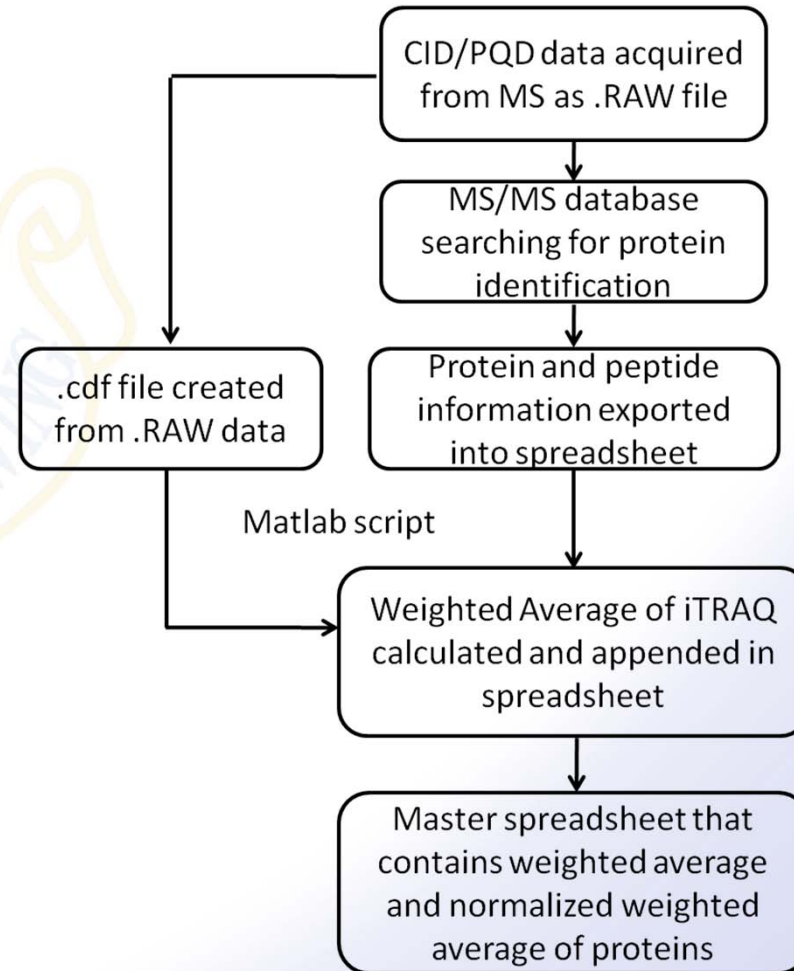




# Data Analysis of iTRAQ Labeled Peptides



- MS/MS data is searched against a database to identify peptides and proteins using SEQUEST in the Bioworks Browser by ThermoFisher.
- This data is exported into a new Excel spreadsheet.
- We have developed a MatLab script that weights the quantitative data using the raw data and appends the results in the original spreadsheet as a new column.
- Excel macros normalize the weighted data and perform statistics as the data is inserted into a master spreadsheet. The master spreadsheet also calculates statistical information for each animal and group.



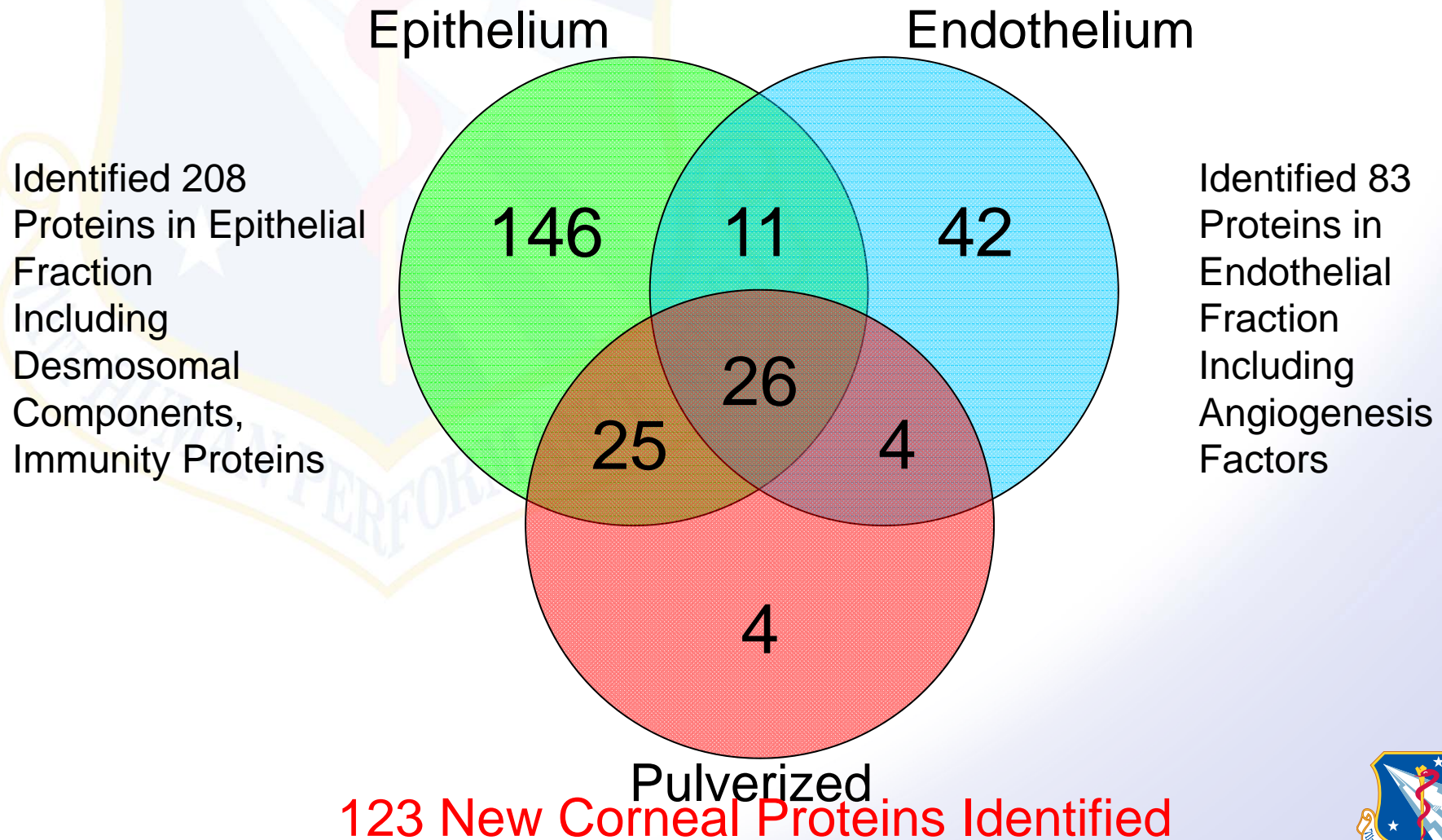
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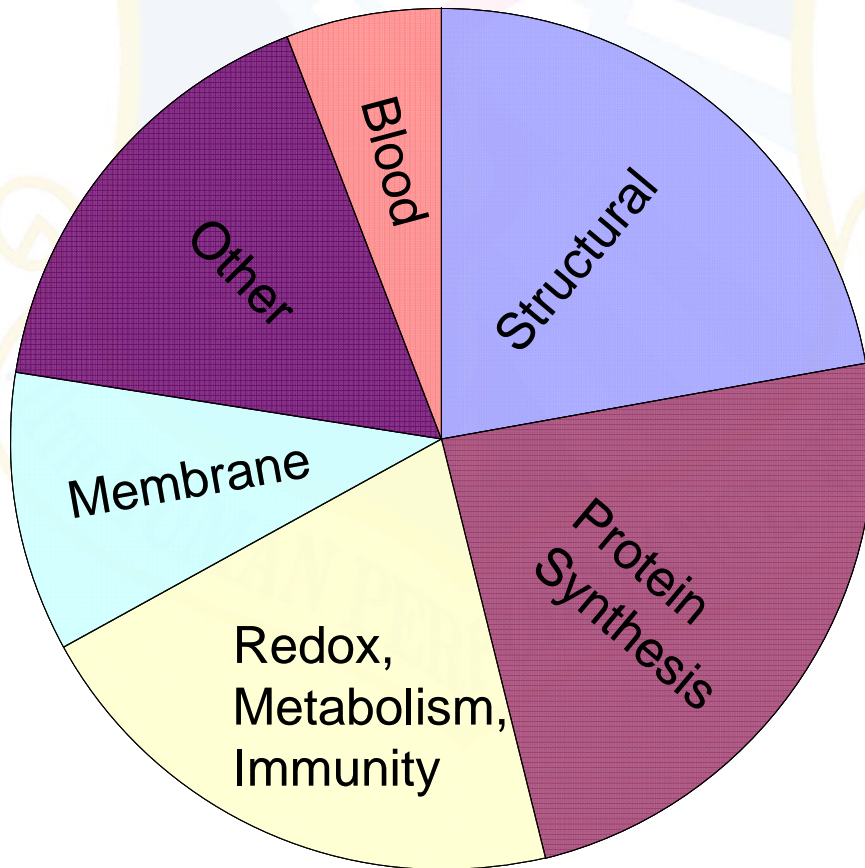


# Identified Corneal Proteins From Method Development

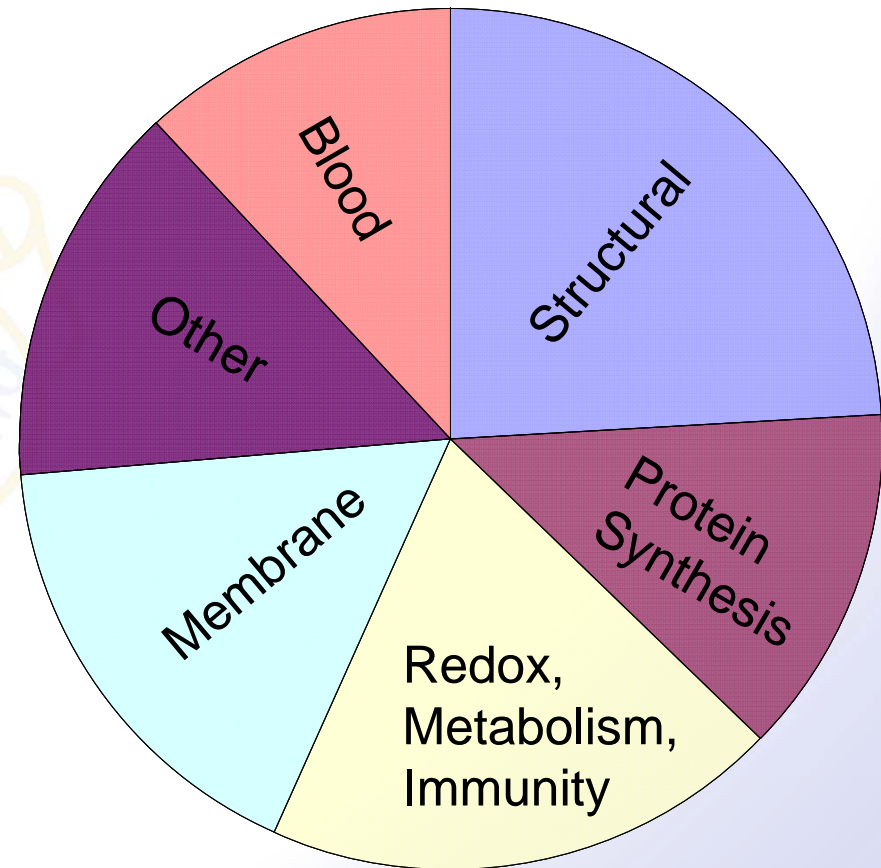




# Functions of Identified Corneal Proteins



Epithelium



Endothelium





# Antibiotic and Corticosteroid Treatment Efficacy



## Study Design

Group	Group ID	n	Challenge	Treatment	Assessment (days) Harvest times (days)
A	Naïve Control	3	None	None	1, 21, 63
B	SM Control	36	SM	PMBST saline	1, 3, 7, 21, 42, 63
C	Treatment	36	SM	PMBST Pred 1%	1, 3, 7, 21, 42, 63

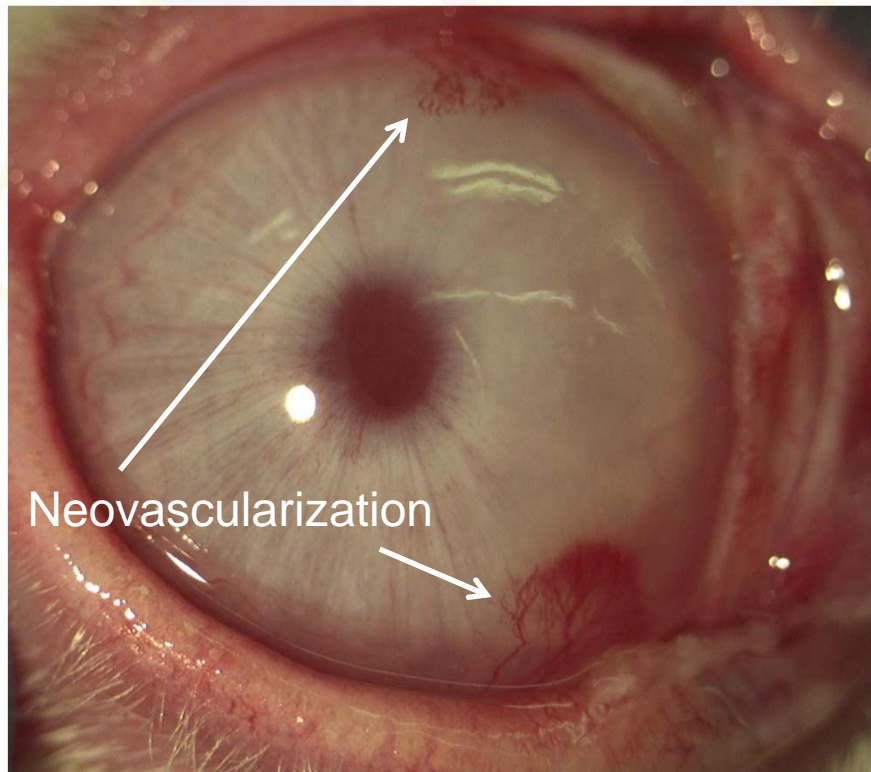
PMBST: Polymyxin B Sulfate and Trimethoprim Ophthalmic Solution







# Clinical Results of SM Exposure and Prednisolone Regimen



- NV does not appear in any animal until day 21.
- At day 21, 7/18 animals in SM group experience NV, while only 1/18 animals in Pred group experience NV.
- At day 42, 5/12 animals in SM group show NV, with only 1/12 exhibit NV in Pred group.
- At day 63 4/6 in SM show NV, with 1 animal showing extensive NV, while 2/6 show minor NV in Pred group.
- Pred group shows significant loss in the corneal thickness compared to SM.





# Experimental Conditions



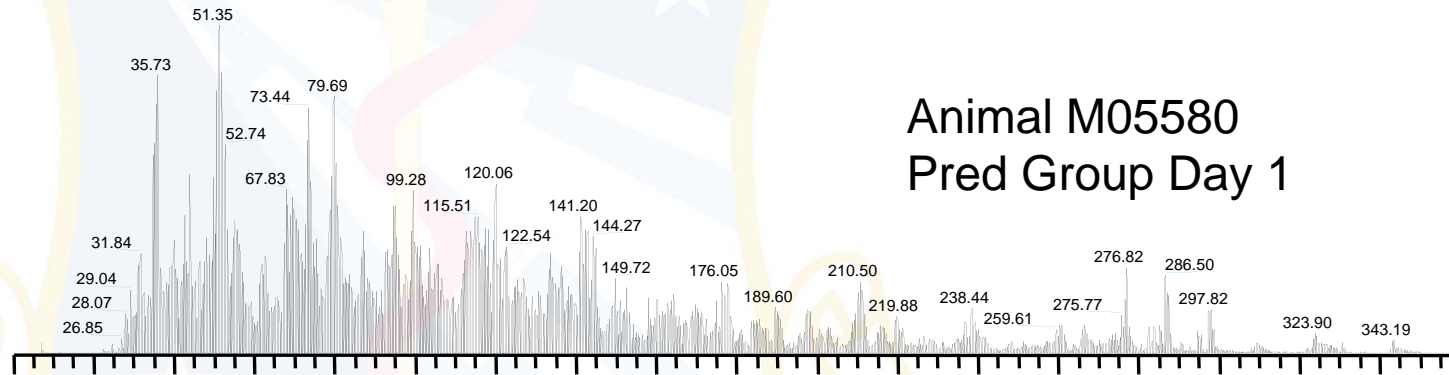
- Approximately 20-80µg of protein were extracted from the epithelium and endothelium. 350-500µg of protein were extracted from the stroma.
- Labeled Epithelial and Endothelial fractions were combined and diluted to a concentration of ~1µg/µl prior to injection.
- Peptides were separated over a 6 hour gradient of increasing concentration of acetonitrile with 0.1% formic acid.
- The mass spectrometer acquires a full scan, followed by CID and PQD dissociation of the top 8 peaks.
- Dynamic exclusion was enabled to limit the number of times a peptide is dissociated to 2.
- ~90,000 scans are acquired per sample run.



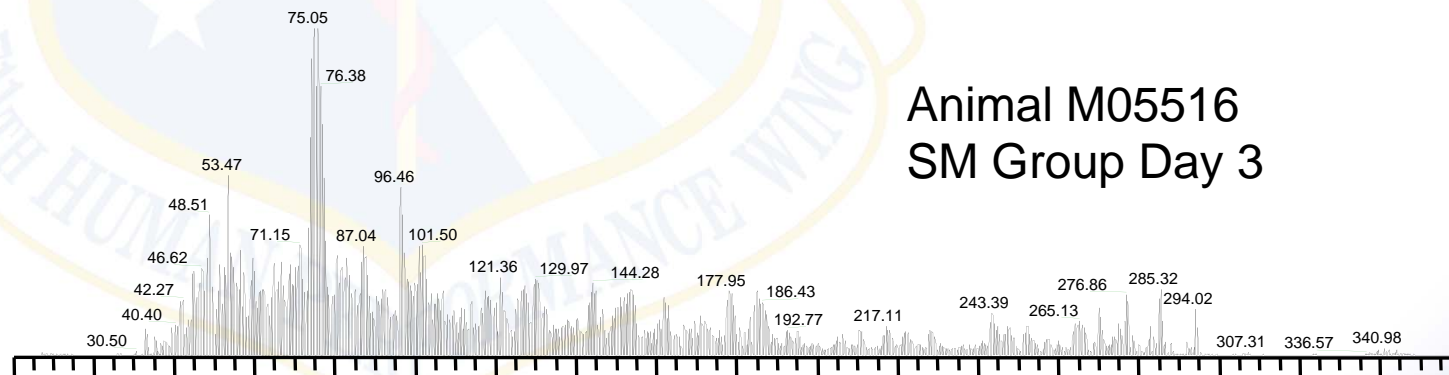




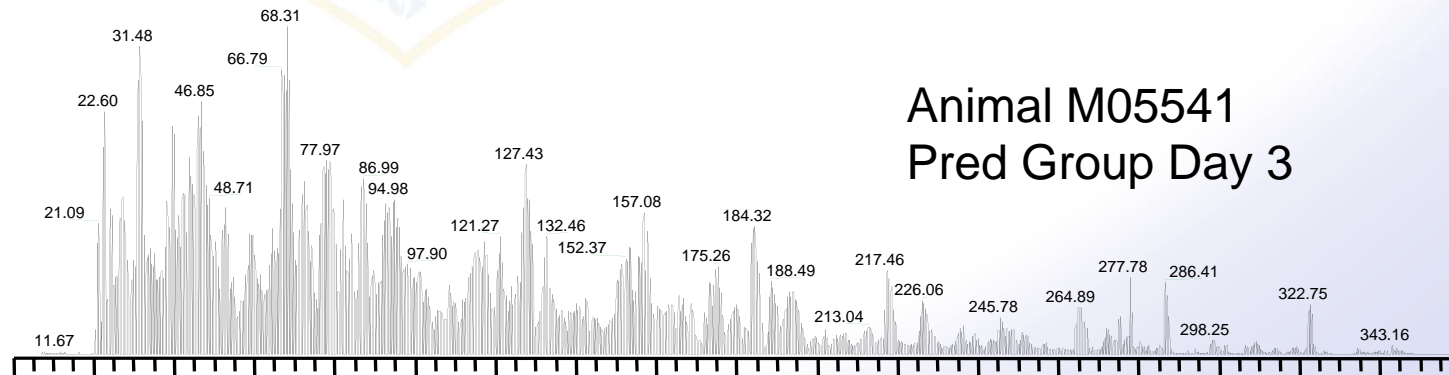
# Base Peak Plots of Selected Epithelial Samples



Animal M05580  
Pred Group Day 1



Animal M05516  
SM Group Day 3



Animal M05541  
Pred Group Day 3

0 60 120 180 240 300 360  
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# Quantitative Protein Results of Epithelia

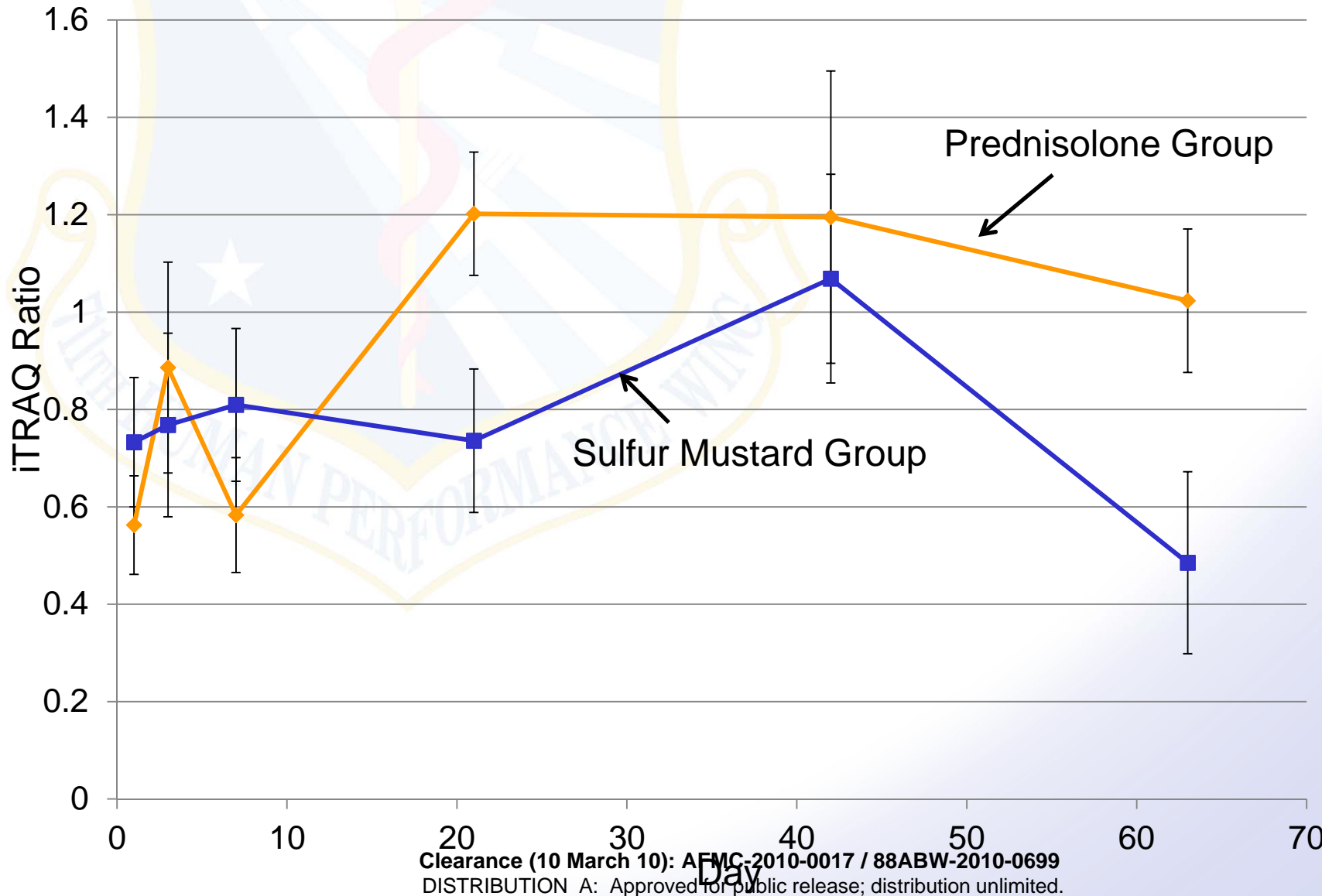


- **Lamin-B1, a protein thought to play a role in the framework of the nuclear envelope, was greatly reduced ( $0.18 \pm 0.03$ ) in Days 1-7 of Prednisolone treated samples**
- **Defensin was elevated to  $12.15 \pm 2.46$  in days 1,3 and 7 of SM group**
- **Keratocan, a protein involved in corneal transparency, is higher in NV animals and than in non-NV animals.**
- **Eukaryotic translation Initiation factor was found to be increased in Pred group NV animals, while remaining at equal levels in all other animals**
- **Alpha 1-antiproteinase, a plasma protein involved in protection from inflammation, is consistently higher in Day 63 than any other time point**





# Pyruvate Kinase Protein Expression of SM and Pred Groups





# Epithelial Proteins Trends from Neovascularized Animals



- Many proteins were found to be reduced when compared with the control cornea
- Proteins involved in glycolysis were reduced across all animals
- Laminin Receptor 1 protein was reduced to  $0.45 \pm 0.19$ . This protein functions plays a role in cell adhesion and activation of signaling transduction pathways
- ATP Synthase was also reduced to  $0.28 \pm 0.10$  in SM animals. The protein was  $2.10 \pm 0.44$  in prednisolone
- Immunoglobulins were found to be increased to  $1.56 \pm 0.20$  in all NV animals
- Transferrin was increased to  $1.83 \pm .56$ , not including 3 animals that showed no transferrin in the control cornea
- Heat Shock Protein 70kDa was also found more elevated on animal M05560 than any other animal, including moderate NV animals.

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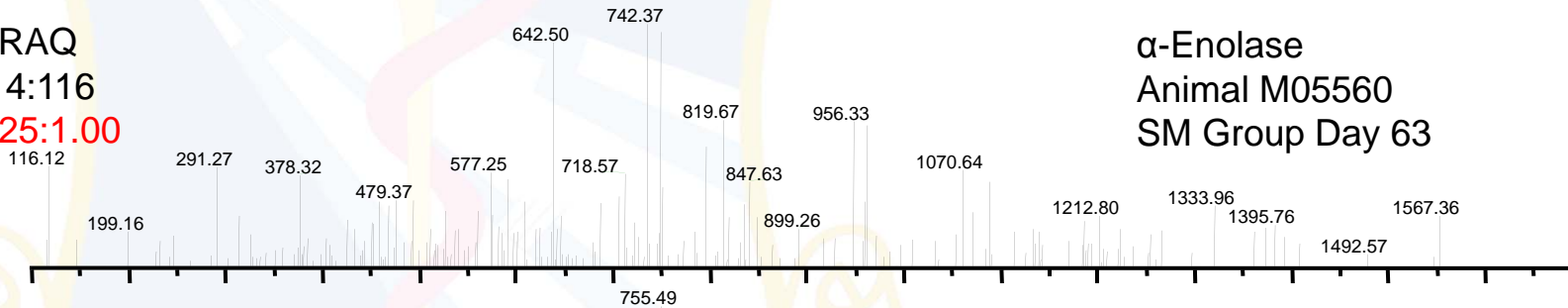


# PQD Spectra of $\alpha$ -Enolase and GAPDH from NV Corneas



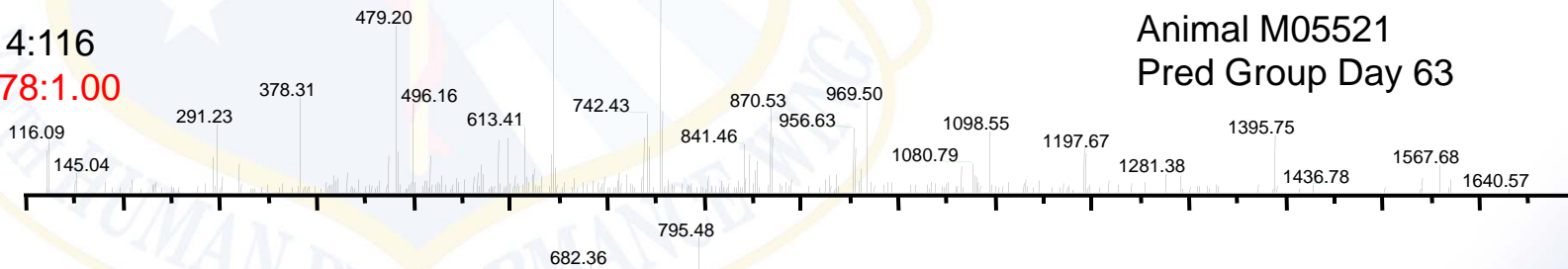
iTRAQ  
114:116  
0.25:1.00

$\alpha$ -Enolase  
Animal M05560  
SM Group Day 63



114:116  
0.78:1.00

$\alpha$ -Enolase  
Animal M05521  
Pred Group Day 63



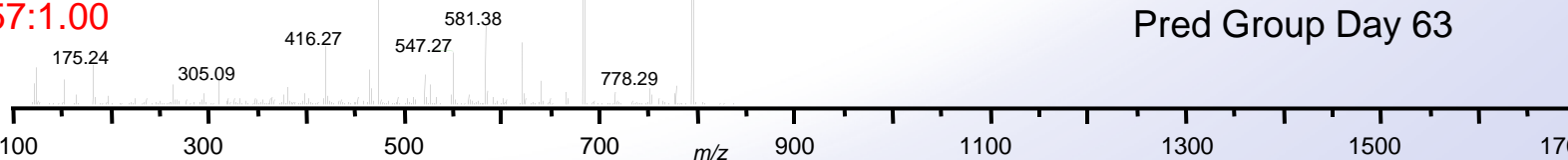
114:116  
0.20:1.00

GAPDH  
Animal M05560  
SM Group Day 63



114:116  
0.57:1.00

GAPDH  
Animal M05542  
Pred Group Day 63







# Protein Averages of NV Epithelia



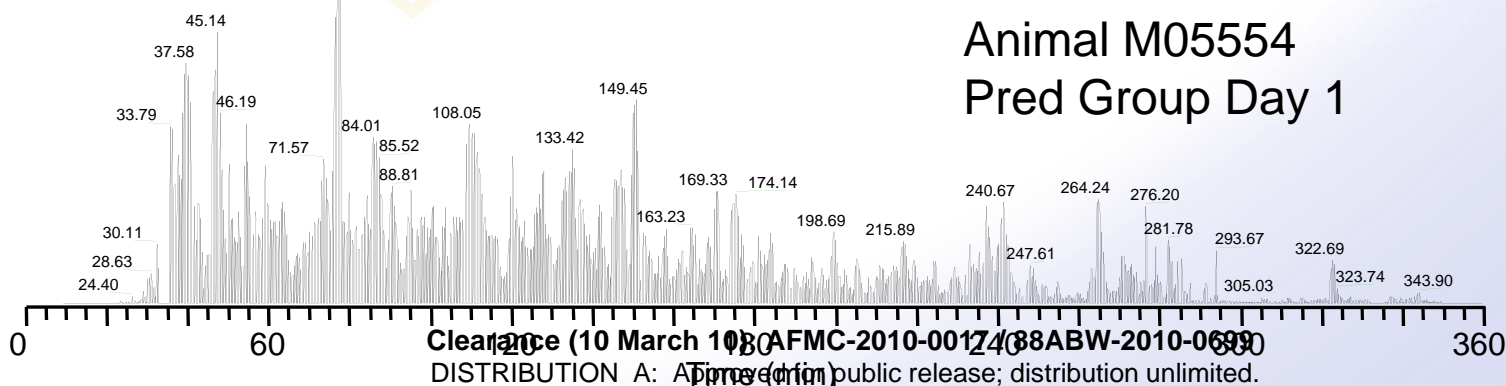
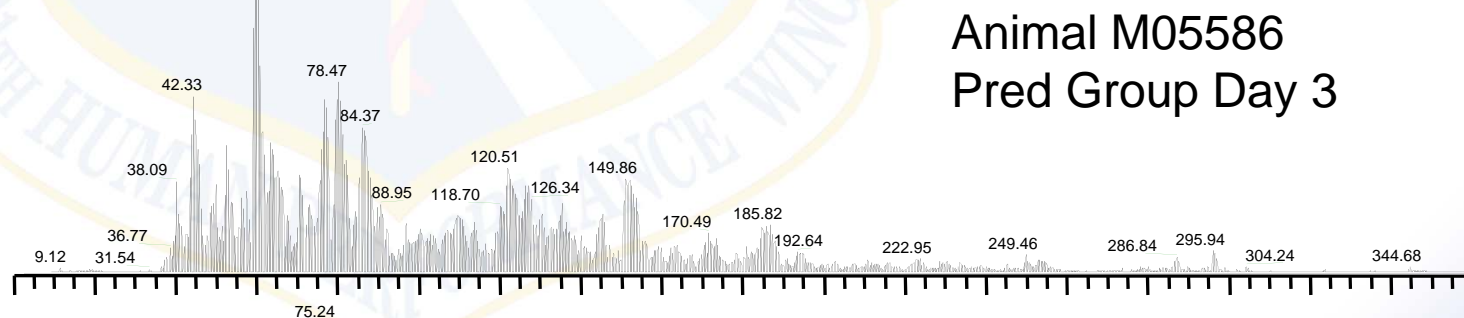
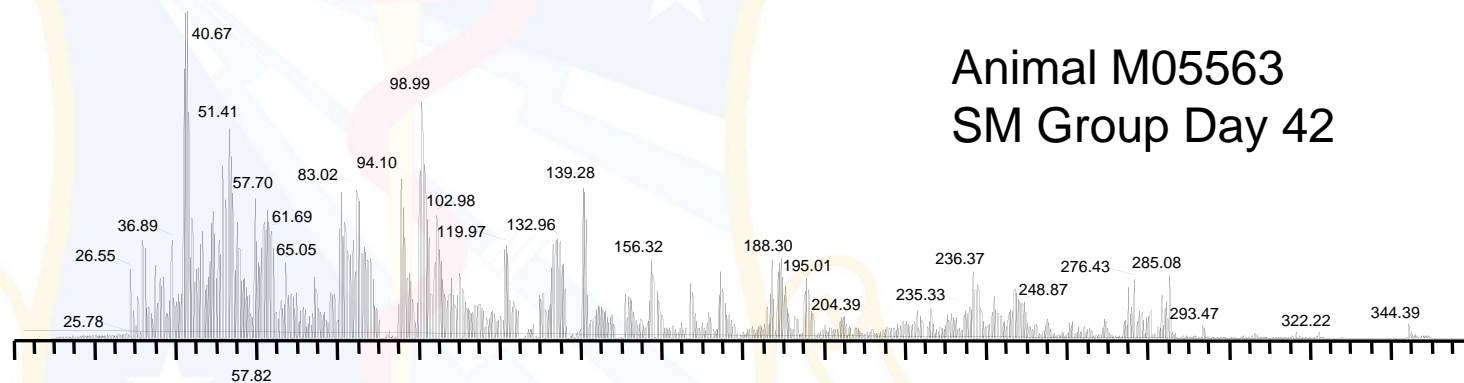
Protein Name	Accession Number	Avg. SM Group	Avg. Prednisolone Group
Pyruvate Kinase	206205	0.49±0.12	1.29±0.27
α-enolase	2661039	0.49±0.09	0.80±0.13
GAPDH	31645	0.41±0.11	0.85±0.20
Phosphoglycerate Kinase	4505763	0.23±0.11	0.42±0.24
Aldehyde Dehydrogenase	2183299	0.47±0.08	0.77±0.11
Lactate Dehydrogenase	187074	0.45±0.12	1.07±0.42
Desmoplakin	1147813	0.78±0.38	0.53±0.27
Junction Plakoglobin	15080189	0.38±0.10	0.91±0.19
Beta-Actin	4501885	0.91±0.27	1.02±0.14

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# Base Peak Plots of Selected Endothelial Samples





# Quantitative Proteomic Results of Endothelia



- Endothelial analysis shows that there was less deviation from the normal than in epithelium
- Lactate Dehydrogenase, a metabolic protein, was found to be reduced in Day 1 (avg= .32) versus later time points (avg= .82)
- Nidogen 1, a structural protein was found to be 1.76 in severe NV, versus normal levels for moderate NV
- Lysyl oxidase like 4, a protein that modulates collagen formation, was present only at later time points
- Cartilage Acidic Protein 1, a protein involved in cell differentiation, was increased at later time points vs. early time points

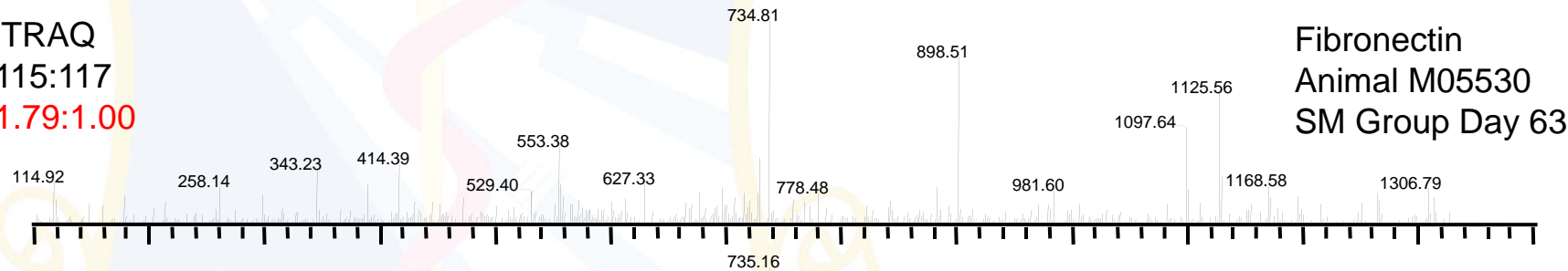




# PQD Spectra of Fibronectin and SPARC-Like 1 from NV Corneas

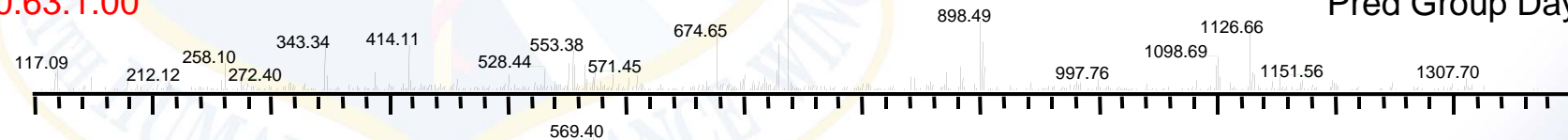


iTRAQ  
115:117  
1.79:1.00



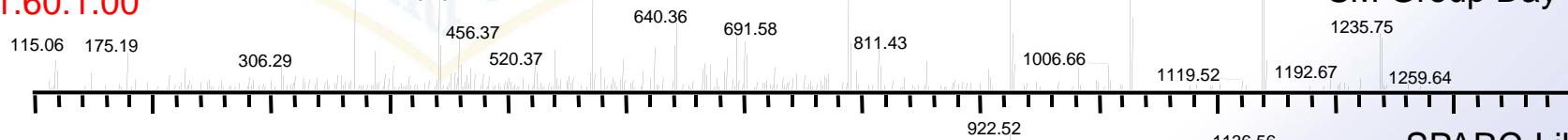
Fibronectin  
Animal M05530  
SM Group Day 63

115:117  
0.63:1.00



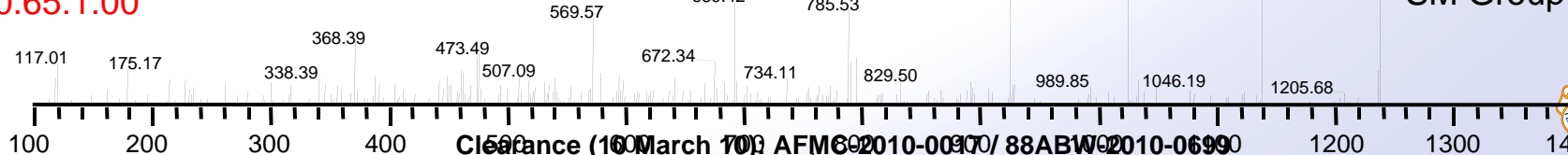
Fibronectin  
Animal M05553  
Pred Group Day 21

115:117  
1.60:1.00



SPARC-Like 1  
Animal M05559  
SM Group Day 63

115:117  
0.65:1.00



SPARC-Like 1  
Animal M05553  
SM Group Day 21





# Protein Averages of NV Endothelia



Protein Name	Accession Number	Avg. SM Group	Avg. Prednisolone Group
Keratocan	5901992	3.07±0.92	3.31±0.33
Fibronectin	31397	1.45±0.14	0.46±0.11
Fibulin-5	19743803	0.83±0.10	0.52±0.10
SPARC-Like 1	21359871	1.26±0.18	0.44±0.07
Lysyl Oxidase Protein Like 4	20177956	2.23±0.38	1.13±0.47
Serum Albumin	126723746	1.88±0.24	1.95±0.31
Transferrin	6175087	2.00±0.41	2.25±0.45
Immunoglobulin	165398	2.54±0.73	2.93±0.25
Thrombospondin-1	40317626	1.77±0.47	0.95 (in only 1 sample)

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# Conclusions

- Removal and separate analysis of the epithelium and endothelium accompanied with long separation times expands the number of identified proteins for quantitation
- Quantitative proteomics using iTRAQ labeling has the ability to profile protein expression across the proteome in the cornea
- Metabolic proteins in the epithelium, including those involved in glycolysis, were found to be more reduced in SM only corneas than prednisolone treated corneas
- Endothelial proteins involved in wound healing and anti-angiogenic proteins were increased across all NV animals
- Prednisolone was found to moderately improve neovascularization of the cornea compared to solely washing in saline

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# QUESTIONS????

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